Temperature fluctuations during development reduce male fitness and may limit adaptive potential in tropical rainforest *Drosophila*

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Abstract

Understanding how organisms tolerate thermal stress through physiological or evolutionary responses is crucial given rapid climate change. Although climate models predict increases in both temperature mean and variance, tolerances are typically assessed under constant conditions. We tested the effect of temperature variability during development on male fitness in the rainforest fly *Drosophila birchii*, by simulating thermal variation typical of the warm and cool margins of its elevational distribution, and estimated heritabilities and genetic correlations of fitness traits. Reproductive success was reduced for males reared in warm (mean 24°C) fluctuating (± 3°C) versus constant conditions but not in cool fluctuating conditions (mean 17°C), although fluctuations reduced body size at both temperatures. Male reproductive success under warm fluctuating conditions was similar to that at constant 27°C, indicating briefly exceeding critical thermal limits has similar fitness costs to continuously stressful conditions. There was substantial heritable variation in all traits. However, reproductive success traits showed no genetic correlation between treatments reflecting temperatures at elevational extremes, potentially constraining evolutionary responses at these ecological margins. Our data suggest that even small increases in temperature variability will threaten species living close to their upper thermal limits, both through direct effects on fitness and by limiting adaptive potential.

Keywords*:* male fitness, development, temperature fluctuations, climate change, adaptive potential, ecological margins

1. Introduction

Climate change research has documented rapid recent changes in mean global temperatures (IPCC, 2014), and has devoted extensive effort to assess the impacts on ecosystems (Walther, 2010), in particular, how the responses of organisms to increased average temperature affects species' persistence in time and space (Parmesan, 2006; Huey et al., 2012; Johnson & Hefin Jones, 2017). However, the effect of the associated trend of increased temperature variability on organismal performance has received far less attention (Lawson et al., 2015), despite evidence that such fluctuations in temperature present a more serious risk to species' persistence than changes to mean temperature alone (Vasseur et al., 2014). This is likely to be especially true in the tropics, where temperature ranges are typically narrower and more stable than in temperate regions (Tewksbury et al., 2008).

Ectotherms are a focus of attention in investigating temperature stress, because (a) they constitute a significant proportion of terrestrial animal species, often with critical roles in ecosystem function (Weisser & Siemann, 2013), and (b) their biochemistry and physiology are directly dependent on ambient temperatures (Deutsch et al., 2008). Temperature is therefore likely to be a major factor limiting their fundamental niche (Angilletta, 2009). Many ectothermic species show latitudinal clines in ecologically-relevant traits, suggesting adaptation to local thermal conditions (Loeschcke et al., 2000; Robinson & Partridge, 2001). Experiments that vary abiotic conditions also expose life-history trade-offs, including negative correlations between reproductive success and stress resistance (Marshall & Sinclair, 2010). Trade-offs such as these can determine species' ecological tolerances, population growth rates and therefore their abundance and geographical distributions (Kimura, 2004; Angilletta, 2009; Edward & Chapman, 2011). However, it remains poorly understood how such trade-offs are determined by the variation in temperature experienced

by wild populations, because laboratory studies have typically tested the fitness costs of thermal stress, and its genetic variance, using constant temperature regimes (Fischer et al., 2011; Colinet et al., 2015), which may have little relevance to those experienced in naturally-varying environments (Mitchell & Hoffmann, 2010; Anderson et al., 2014). The heritability of a trait can also vary substantially depending upon the environment, making it important to estimate genetic variance and trait correlations under ecologically realistic conditions (Hoffmann & Merila, 1999), in order to determine the potential for evolutionary responses to climatic change (Hoffmann & Sgro, 2011).

Where ecological and evolutionary responses to varying temperatures have been estimated, research has tended to focus on extreme conditions, measuring traits such as heat stress survival or cold shock/chill coma (Hoffmann et al., 2003; Griffiths et al., 2005; Bridle et al., 2009; Bozinovic et al., 2011). However, temperature variation within generally existing temperature ranges may have significant sub-lethal effects on fitness-related traits (Kjaersgaard et al., 2013; Manenti et al., 2014). Also, cosmopolitan species with widespread distributions (*e.g. Drosophila melanogaster, D. subobscura*) have commonly been used to assess such thermal impacts, largely because of their laboratory tractability, although even closely related species often show contrasting temperature optima and thermal niche widths (Kellermann et al., 2012). In particular, tropical species are expected to be more thermally specialised due to the narrower range of climatic conditions they experience and may therefore be more sensitive to changing thermal conditions (Deutsch et al., 2008). Additionally, unlike temperate ectotherms (where performance optima are typically found at temperatures higher than mean habitat temperatures), tropical ectotherm performance is maximised at their mean habitat temperature, suggesting greater vulnerability to increased fluctuations at high temperatures, even if the mean does not change (Amarasekare & Johnson, 2017). However, where species are distributed across thermal gradients within their

range (*e.g.* elevational or latitudinal gradients), there may be both genetic variation in thermal sensitivity and adaptation to local conditions that can be related to local and global limits to distributions (Ghalambor et al., 2006; Bridle et al., 2009).

Investigations of how thermal environments influence reproduction in ectotherms, including *Drosophila*, have largely tested the effect of temperature on adult performance and behaviour (Katsuki & Miyatake, 2009; Onder, 2009; Terblanche et al., 2010; Bozinovic et al., 2011) or how contrasting constant temperature regimes influence development and subsequent adult fitness (Nunney & Cheung, 1997; Hoffmann et al., 2003; Fragata et al., 2016). However, testing how fluctuating thermal environments affect egg-to-adult development and their consequences for adult fitness is crucial, because such early life stages in holometabolous insects have more restricted thermoregulatory capacity, due to the size and limited mobility of larvae, and their rapid growth (Feder et al., 1997; McMillan et al., 2005). Accordingly, several adult morphological traits in insects, such as wing size (a proxy for overall body size) and stress-tolerance (*e.g.* heat knockdown, chill coma recovery, starvation and desiccation resistance), decrease with exposure to fluctuating temperatures during development (Kjaersgaard et al., 2013; Manenti et al., 2014). Such a consistent reduction in adult size is significant because male body size correlates strongly with mating success and lifetime reproductive success in *Drosophila* (Partridge & Farquhar, 1983). Mating success itself is also significantly reduced by high thermal stress (>36°C) on adult males (Onder, 2009) and latitudinal clines in male mating-related traits in *D. melanogaster* suggest that abiotic conditions drive selection on remating (Chahal et al., 2013). What is not known, however, is: (a) whether similar fitness effects are induced by temperature variation that is routinely experienced within an ecological distribution; and (b) whether exposure to such conditions during development affects the genetic variance and therefore adaptive potential of these

traits. Such data are crucial in understanding the causes of existing ecological margins, and the potential for future evolutionary responses to buffer the consequences of climate change.

In this study, we exposed flies from isofemale lines of the tropical rainforest fly *Drosophila birchii*, collected across this species' elevational range (20–1100 metres above sea level), to contrasting thermal treatments during their egg-to-adult development, that include those found at this species' ecological limits. These treatments included different mean temperatures (warm or cool), with temperature either held constant or fluctuating (± 3°C) around the mean. The effect of these treatments during early life stages on adult male reproductive traits (number of successful matings, offspring productivity of each individual mating, total offspring productivity) and wing centroid size were compared to determine: (i) the consequences for male reproductive fitness of thermal variation experienced at local ecological limits, (ii) genetic variation in the effects of the treatments on isofemale lines collected from different elevations, and (iii) heritability and genetic correlations between traits measured in each temperature regime.

2. Materials and methods

(a) Origin of isofemale lines

Drosophila birchii (Dobzhansky & Mather; Drosophilidae) is a tropical fruit-fly species restricted to rainforest habitats of north-eastern Australia and Papua New Guinea (Schiffer et al., 2007). Laboratory isofemale lines (hereafter 'lines') for Experiments I and II were derived from field caught *D. birchii* females collected at sites along two elevational gradients at Mount Edith (17°6'S, 145°38'E) and Mount Lewis (16°35′S 145°17′E), in north-eastern Queensland, Australia, in 2011. The elevation of the Mount Edith collection sites ranged from ~600–1100 metres above sea level over a distance of 4.3 km. Mount Lewis sites ranged from ~20–900 metres in elevation over a distance of 16.3 km. For Experiment III, *D. birchii* were collected at sites at Paluma (18°56′S 146°10′E) and Mount Lewis, in 2016. The Paluma gradient ranged from ~70–900 metres over a distance of 3.4 km.

A 'mass-bred' stock was founded by combining 10 male and 10 female flies from each line, to act as a genetically mixed background population from which females could be derived to test focal males against. The same mass-bred stock was used for Experiments I and II, while a new stock was founded for Experiment III using the same procedure, but comprising the isofemale lines in Experiment III. Experiment I and II took place after ~75 laboratory generations. For Experiment III, lines were established for ~12 generations before the experiment commenced. Further information about the establishment of lines can be found in supporting material (SM).

145 ***(b) Experiments I and II: The effects during development of thermal conditions at warmer***
146 ***and cooler ecological limits on male fitness***

147 In Experiment I, we estimated adult male remating ability, productivity and body size after
148 exposure to warm (mean 24°C) constant and fluctuating (± 3°C) conditions during egg-to-
149 adult development. In Experiment II, we measured the same traits for males that had
150 developed in cool (mean 17°C) constant and fluctuating (± 3°C) conditions. Due to logistical
151 constraints and the varying development times under each thermal regime, the warm and cool
152 experiments were not synchronised and are therefore not directly comparable.

153 Thermal conditions for each treatment were determined using hourly temperature field data
154 collected using Tinytag data loggers from four sites along the Mount Lewis gradient (with the
155 largest total change in elevation) from February 2010 to June 2012. Mean daily temperatures
156 of ~24°C ± 3°C were recorded at the warm, low elevation sites and a mean daily temperature
157 of ~17°C with relatively small fluctuations (± 1°C) at cold, high elevation sites (see SM for
158 detailed field temperature data). The four laboratory treatments therefore include two thermal
159 regimes typical of those at the local ecological limits of *D. birchii* (warm fluctuating and cool
160 constant), as well as two treatments with the same mean temperatures as these but with
161 fluctuations equal to that in the contrasting thermal treatment (warm constant and cool
162 fluctuating) (see Table 1). Such a design disentangles the effects of increased mean
163 temperature vs. increased temperature fluctuation. The fluctuating regimes mimicked a
164 natural diurnal cycle with the higher temperature lasting 6-hours during the light period and
165 the lower temperature for 6-hours during the dark period. Between these periods the
166 temperature would ramp over 6-hours between the high and low temperatures (see SM for
167 further details).

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Table 1.

Experiment I:	Warm Constant	Warm Fluctuating
Low Elevation	24°C	21 – 27°C
Experiment II:	**Cool Constant**	**Cool Fluctuating**
High Elevation	17°C	14 – 20°C

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171 **Table 1**. Four thermal regimes that focal males were exposed to during development in
172 Experiment I and II (treatments that replicate natural thermal regimes are in **black,** paired
173 treatments of matching mean temperature are in grey).

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For each of the experiments, we used 20 lines (5 high, 5 low elevation from each of the Mount Edith and Mount Lewis gradients). Twenty focal males per line were assayed per treatment (n= 1600 males). Prior to both experiments, lines were maintained in 40 ml vials at constant 20.5°C control temperature, outside of the range of any of the experimental temperature treatments, under a 12:12-h light:dark cycle at 60% relative humidity (RH) for at least two generations to standardise maternal environmental effects.

Five days after eclosion, the parents of the experimental flies were placed in vials containing 10ml of fly medium for 4 days under low-density conditions (5 males and 5 females per vial) to minimise larval competition. Fly food batches were randomised throughout the experiment. After four days, parents were discarded and pupation card was inserted. Vials were then transferred to temperature treatments (Snijders Labs 780-l Insect Chambers). Light intensity and ambient humidity were standardised (12:12-h light:dark cycle, 60% RH) and vial position randomised daily. Female flies from the mass-bred population were reared using the same procedure at a constant temperature that matched the mean temperature for cold or warm treatments (Experiment I: 24°C, Experiment II: 17°C).

Less than 12 hours after emergence, virgin males from the isofemale lines and virgin females from the mass-bred population were collected under CO_2 anaesthesia. Flies were held in single-sex, low-density (max. 10 individuals) holding vials containing 5ml of *Drosophila* medium at constant 20.5°C. Six days after collection, each focal male was placed in a vial with three virgin mass-bred females (also six days old) for 24 hours under the same conditions and allowed to mate. Each male was then removed and preserved in 100% ethanol, together with males from the same line and treatment. Any focal males found dead were excluded from the study. After removing the male, females were transferred to individual vials and allowed to lay for 5 days. Pupation card was then added to each vial and offspring

emergence estimated at 20.5°C. Female *D. birchii* rarely re-mate within a 24-hour period (*pers. observation*), so we assume that the number of females that produced offspring from a given male represented the total number of matings. The number of offspring produced by each female was used to obtain the following measures of male reproductive success: (i) number of successful matings per male (from 0–3 females) (ii) number of offspring from each individual mating and (iii) total productivity of offspring per focal male.

(c) Morphometric analysis of wing size

Wing size variation of focal males was analysed using a protocol described by Griffiths et al. (Griffiths et al., 2005). All wings were randomised and a sub-sample of wings was re-photographed and landmarked each measurement session to check error variance (± 3% of total centroid variation). See SM for detailed information.

(d) Experiment III: Testing the effects of warm temperature treatments

In Experiment III, we tested whether variation in male reproductive success in Experiment I was due to exposure to fluctuating conditions *per se* or to exceeding key thermal thresholds in the warm fluctuating treatment. This experiment repeated the warm fluctuating treatment used in Experiment I (24 ± 3°C) along with two additional temperature regimes at the limits of this treatment: 21°C constant and 27°C constant. All other methods and measures of male mating success were identical to Experiment I except that the control temperature for rearing background females, for mating and female egg-laying was a constant 24°C (the mid-point temperature of the treatments). In Experiment III, we used 16 lines (4 high, 4 low elevation from each of the Paluma and Mount Lewis gradients). We tested 10 focal males per line per treatment (n= 480).

(e) Statistical analyses

We fitted general linear mixed models to test for effects of thermal regime on measures of male reproductive success (offspring per mating, total offspring) and body size in each experiment, and to estimate genetic variation in these traits. Separate models were fitted for each experiment and trait. All analyses were performed using the package *lme4* in R v 3.4.0 unless otherwise specified. In each case, treatment was modelled as a fixed factor. The random effects of line nested within elevation (high or low) and transect (Mt Edith and Mt Lewis in Experiments I and II; Paluma and Mt Lewis in Experiment III) were included to partition variation due to these factors. The significance of each factor in the model was determined using likelihood ratio tests to compare the full model with a model where that factor had been removed. All data were untransformed after testing for a normal distribution.

For Experiments I and II, we used the among-line variance to estimate broad-sense heritability (H^2) of fitness and body size traits. To compare genetic variance and heritabilities across the different rearing environments, we ran models for each treatment separately, with line included as a random factor and with no fixed factors. Other random factors included in initial models (transect and elevation) did not improve model fit and were excluded. Within and between line variance components (V_w and V_b respectively) were estimated from models using REML. We used these to calculate H^2 following the method of Hoffmann and Parsons (Hoffmann & Parsons, 1988), calculating the inbreeding coefficient (F_t) according to Falconer and Mackay (Falconer & Mackay, 1996). We evaluated whether H^2 was significantly different from zero by comparing models with and without the random effect of line, using a likelihood ratio test (see SM). However, H^2 was not calculated for Experiment III because the relatively low number of isofemale lines and males within lines limited our power to estimate these components of variation.

The isofemale line means for each trait in Experiments I and II were used to estimate broad-sense genetic correlations between traits, and of the same trait across different rearing environments. We used linear regression to obtain the Pearson product moment correlation (R^2) between pairs of trait means within each treatment (genetic correlations between traits), or of the same trait across each pair of rearing treatments (cross-environment genetic correlations).

3. Results

(a) Effect of temperature fluctuation on male reproductive success and body size

Males reared under warm-fluctuating (24 ± 3°C) conditions on average obtained ~22% fewer successful matings, producing ~27% fewer offspring per mating, with a ~35% reduction in total number of offspring compared to those reared under constant conditions at the same mean temperature. Wing centroid size, a proxy for overall body size (James et al., 1995), decreased by ~2% under warm fluctuating conditions vs. warm constant conditions. A similar effect of fluctuating rearing conditions on body size was seen at cool (mean 17°C) conditions. However, fluctuation did not significantly affect any of the measures of male reproductive success at this lower temperature (Figure 1). There was significant among-line variation for all measured traits. There was no indication of locally adapted responses to temperature treatments in either experiment, given that neither transect or elevation of origin of lines was associated with significant variation in any trait (Table SM2).

(b) Comparing warm fluctuating treatment to constant lower and higher temperatures

Results from Experiment III suggest that the 24°C fluctuating treatment reduced male reproductive success as much as a constant 27°C regime, when compared to the higher mean number of offspring observed at constant 21°C (Figure 2; Table SM4). There was a ~30% decrease in mean total offspring between 21°C constant and 24°C fluctuating and ~28% between 21°C and 27°C constant. Wing centroid size differed significantly between all treatments with a ~10% reduction between 21°C constant and 24°C fluctuating and ~12% decrease between 21°C and 27°C. There was also a small decrease between 24°C fluctuating and 27°C constant (~3%).

(c) Trait heritabilities and genetic correlations between traits and rearing environments

Among-line variance for all traits in all rearing environments were highly significant (Table 2). Heritabilities did not differ between rearing environments for any trait (as indicated by overlapping 95% CIs).

Within each rearing environment, there were strong genetic correlations between the two traits relating to male reproductive success. This perhaps is unsurprising given that total offspring is used in the calculation of offspring per mating. However, genetic correlations between each of the reproductive success traits and wing size were not significantly different from zero in any environment except cool-constant, indicating body size does not correlate with male fitness. However, our power to detect such differences was relatively low due to the use of line means (Table SM5). Cross-environment genetic correlations were generally high and highly significant, with the exception of the reproductive success traits between the warm fluctuating and the cool constant conditions that replicate the regimes found at *D. birchii*'s elevational limits (Table 3).

318 Table 2.

Regime		Trait	V_b	V_b 95% CI	V_w	V_w 95% CI	H^2	H^2 95% CI	p
Warm: Mean 24°C	Constant	Total offspring	689.3	201.5, 1381.5	5192.3	4483.9, 5966.7	0.121	0.044, 0.194	***
		Offspring per mating	114.2	40.7, 218.4	560.7	484.8, 638.5	0.174	0.080, 0.263	***
		Wing size	1719	747.4, 3158.2	2240	1871.7, 2658.9	0.448	0.294, 0.560	***
	Fluctuating	Total offspring	1141	438.4, 2263.0	4525	3879.0, 5197.0	0.208	0.105, 0.313	***
		Offspring per mating	133.5	41.5, 251.4	673.3	579.3, 772.2	0.171	0.069, 0.253	***
		Wing size	1393	509.4, 2651.4	3773	3170.0, 4468.3	0.278	0.143, 0.384	***
Cool: Mean 17°C	Constant	Total offspring	899	335.3, 1656.8	2894	2506.8, 3327.4	0.244	0.122, 0.343	***
		Offspring per mating	200.4	75.2, 384.8	720.5	616.7, 829.3	0.224	0.112, 0.327	***
		Wing size	2194	974.8, 3896.2	2751	2336.0, 3203.1	0.457	0.304, 0.566	***
	Fluctuating	Total offspring	748.4	244.6, 1471.3	3426.7	2983.2, 3934.0	0.185	0.078, 0.281	***
		Offspring per mating	179.7	60.5, 727.6	855.3	727.6, 984.9	0.179	0.079, 0.438	***
		Wing size	2718	1151.1, 4788.7	3181	2718.5, 3689.4	0.475	0.307, 0.582	***

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320 **Table 2.** Estimates of broad-sense heritability (H^2) of traits of males from each rearing
321 environment, calculated from components of variance among (V_b) and within (V_w) isofemale
322 lines. F_t the inbreeding coefficient was calculated as 0.485 for all estimates (Eqn 2; see SM).
323 95% CIs on variance components and heritability estimates are from 1000 bootstrap
324 simulations. *P*-values are from model comparisons using likelihood ratio tests to evaluate
325 whether H^2 was greater than zero (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$). H^2 was significantly
326 greater than zero for all traits and rearing treatments after correcting for multiple
327 comparisons.

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329 Table 3.

Rearing environment 1	*Rearing environment 2*	*Trait*		
		Total offspring	*Offspring per mating*	*Wing size*
24°C Constant	24°C Fluctuating	**0.529** ***	**0.483** ***	**0.721** ***
24°C Constant	17°C Constant	**0.337** **	**0.311** ***	**0.467** ***
24°C Constant	17°C Fluctuating	**0.298** ***	**0.247** *	**0.402** **
24°C Fluctuating	17°C Constant	0.003	0.123	**0.661** ***
24°C Fluctuating	17°C Fluctuating	**0.190** *	**0.322** **	**0.604** ***
17°C Constant	17°C Fluctuating	**0.510** ***	**0.470** ***	**0.733** ***

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331 **Table 3.** Genetic correlations (r_G) between traits of males across each pair of rearing
332 environments, estimated from the cross-environment correlations of the isofemale line means
333 for each trait. Showing probabilities (p) that $r_G = 0$ (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).
334 Correlations where r_G is significantly greater than 0 (corrected for multiple comparisons) in
335 bold.

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4. Discussion

Predicting the ecological consequences of climate change depends on understanding how organisms will respond to increasing temperature variability, as well as the effects of increased mean temperatures (Johnson & Hefin Jones, 2017). This study demonstrates that temperature fluctuations during egg-to-adult development, typical of those observed at the warmer (low elevation) ecological limits of the tropical rainforest species *D. birchii*, substantially reduces fitness in males. However, this effect was not significant at temperatures corresponding to those found at the cooler (high elevation) limits of this species' range. In addition, the impact of fluctuations at the warmer mean temperature was comparable to that seen with rearing in a constant high temperature regime (27°C). These data indicate that exposure to heat stress during egg-to-adult development, even for short and predictable periods, causes a reduction in adult male fitness equivalent to that caused by continuous exposure to such temperatures (Figure 2, Table SM4). Such strongly deleterious effects of even small increases in temperature variability, even without a large increase in mean temperatures, suggest that tropical ecosystems will suffer negative consequences of climate change sooner and more severely than is currently predicted.

Differences in reproductive output between the constant and fluctuating regimes may result from costs associated with plastic responses to developmental temperature (Angilletta, 2009). However, our experiments show that these fitness costs (at least in males) are only significant at the warmer margins of a species' distribution, in comparison to the cooler margins. Thermal plasticity, despite reducing heat stress in ectothermic species, is often unable to completely compensate for the impacts of temperature variability (Gunderson et al., 2017), and although larvae and pupae of *Drosophila melanogaster* survive at much higher temperatures (>35°C) in natural environments (Feder et al., 1997), their restricted mobility

limits the scope for behavioural thermoregulation. By contrast, adults can disperse to seek appropriate microclimates and minimise thermal stress (Kearney et al., 2009).

Sperm formation is particularly heat sensitive (David et al., 2005), with temperature thresholds at which males can produce sperm being narrower than those that allow survival or limit the expression of other stress-related traits (Jorgensen et al., 2006). In male insects, it begins during larval stages, so that mature sperm are produced by the time of adult eclosion (Dumser, 1980). Heat stress can directly affect the viability of gametes or may generate a trade-off between sperm quantity and performance and investment in heat shock proteins (Hsps) (van Lieshout et al., 2013), that increase thermotolerance in larvae and pupae (Feder et al., 1996). Typically, trade-offs between temperature fluctuation and fertility or productivity have been assayed using female reproductive output (Dillon et al., 2007; Marshall & Sinclair, 2010; Manenti et al., 2014; Colinet et al., 2015), given the relative ease of measuring this trait in females (Flatt, 2011) and the assumption that the reproductive potential of males contributes less to population demography (Markow, 1996). Compared to other insects, sperm production is especially energetically costly in many *Drosophila* and may be a limiting factor in male fitness (Bjork et al., 2007), and increases in Hsp expression under thermal stress can be particularly localised in reproductive cell types such as the testes (Michaud et al., 1997). Thermally-stressed males can regain fertility if they are moved to benign temperatures, suggesting that damage to male germ cells is not permanent (David et al., 2005). Our study tested males 5–6 days after being removed from the experimental temperature treatments, indicating that fluctuating thermal stress during egg-to-adult development has persistent effects on male mating success. This means that, even if reversible, the losses of reproductive capacity observed will remove a substantial proportion of lifetime reproductive success of *D. birchii* (Jorgensen et al., 2006). In addition, the increase in the proportion of males failing to produce any offspring when reared in the warm

391 fluctuating (~18% of males) compared with the warm constant (~5% of males) environments
392 (Table SM3) suggests heat-induced male sterility. Again, this is likely to increase
393 substantially under the more variable temperature regimes predicted by climate change.

394 Our data are consistent with the non-linear impacts of fluctuating temperatures predicted by
395 the asymmetric thermal performance curves (TPCs) for ectotherms, with sharp decreases
396 anticipated when fluctuations exceed critical thresholds (Colinet et al., 2015). In *Drosophila*
397 *simulans*, a cosmopolitan species with a wide latitudinal distribution, temperature variability
398 causes a small but significant decline in female productivity, but only with much larger
399 fluctuations (13–28°C)(Manenti et al., 2014). In *Aedes aegypti* mosquitoes, small diurnal
400 fluctuations (7.6°C) during development produced only a negligible or small positive effect
401 on female reproductive output, while only much greater diurnal temperature variations
402 (18.6°C) led to significant decreases (Carrington et al., 2013). By contrast, temperature
403 fluctuations of a similar magnitude as we used (18 ± 3°C) actually improve performance in
404 several life-history traits in the male yellow dung fly (*Scatophaga stercoraria*), although
405 larger fluctuations (± 6°C) resulted in smaller male body size (Kjaersgaard et al., 2013). Zeh
406 et al. (Zeh et al., 2014) found temperature fluctuations (~8°C) reduced sperm number and
407 viability in tropical pseudoscorpions (*Cordylochernes scorpioides*) under predicted climate
408 warming scenarios, although no assessments of mating success or reproductive output (or
409 genetic variation in these traits) were made. These studies also only assayed laboratory
410 populations derived from a single location (in relation to elevation or latitude). This means
411 they do not test whether population divergence across geographical ranges is likely to affect
412 costs associated with fluctuating temperatures.

413 Latitudinal clines in responses to thermal stress have been observed in *D. subobscura*
414 (Porcelli et al., 2017). However, there is little evidence for local adaptation in male thermal

stress across shorter distances, even when – as in this case - thermal regimes vary at similar magnitudes locally to those observed across a given species' latitudinal range. In this study, we found no evidence for locally adapted responses, consistent with most other studies of *D. birchii* along elevational gradients (Bridle et al., 2009; O'Brien et al., 2017). However, we observed a highly significant among-line variation for male reproductive success and body size, with broad-sense heritabilities (H^2) ranging from 0.121 –0.244 for reproductive traits and 0.278 – 0.475 for body size (Table 2). Such heritable variation can change substantially depending on environmental conditions, meaning the potential for evolutionary responses varies across environments (Hoffmann & Merila, 1999). In this experiment, there was a trend toward higher H^2 in the cool (mean 17 °C) vs the warm (mean 24 °C) rearing environments, and although not significantly different, it is consistent with several studies showing reduced genetic variance at warmer temperatures in ectotherms, indicating that climate warming may reduce evolutionary potential (Kelly et al., 2012; Schou et al., 2014; Kristensen et al., 2015). However, the effect of increased temperature variability on heritable variation has received far less attention (Schou et al., 2014).

Our results suggest that temperature fluctuations can affect genetic variance in these traits, particularly at warm rearing temperatures, although the direction of this effect varied across traits. While such differences were not significant, H^2 for total offspring was higher in the fluctuating warm treatment than in the constant warm treatment, whereas the reverse was true for body size. However, within the cool treatments, H^2 for all traits showed little difference between constant and fluctuating conditions. Cross-environment genetic correlations showed that reproductive traits were not significantly associated between the temperature regimes typical of the high and low elevation limits of *D.birchii* (*i.e.* warm-fluctuating and cool-constant; Table 3), suggesting little overlap in the genes underlying variance in reproductive success across these two environments (Charmantier & Garant, 2005). This suggests that

what is now favoured in cool, stable environments will not predict male reproductive performance under warmer, fluctuating conditions. Further research, incorporating a broad range of species and traits, is needed to assess whether the effect of fluctuation on genetic variance at warm temperatures observed here is a general phenomenon in ectotherms, and to test for consistent patterns across different categories of traits in the direction of the effect.

As well as direct effects of thermal regime on sperm production and viability, the effect of fluctuating conditions may affect mating success, given that larger body size is generally correlated with courtship and/or mating advantage in *Drosophila* (Partridge & Farquhar, 1983; Fasolo & Krebs, 2004). In our results, body size was reduced in males reared under fluctuating conditions at both warm and cool temperatures. However, the reductions in body size were comparatively small (although significant) and the decline in mating success with temperature fluctuation was only observed in the warm environment, yet only males reared in cool constant conditions showed any significant correlation between mean body size and mating and/or productivity (Table SM5). Other impacts of thermal stress on male courtship could include changes to cuticular hydrocarbons that are key chemical cues used in mate choice in *Drosophila* (Markow & Toolson, 1990) or may arise from indirect effects through impairment of traits such as ability to fly (Krebs & Thompson, 2005). Female sexual selection on such traits could therefore determine the consequences of thermal stress for male fitness in nature (Partridge et al., 1987). Future experiments should examine the effect of rearing conditions on female preference, to assess whether they mitigate or exacerbate the effects on male fitness that we observed.

The substantially reduced male mating success found in the warm fluctuating treatment occurred within a thermal regime that simulated natural conditions for a single generation at the low elevation margin of *D. birchii*'s distribution. What is surprising is the size of these

effects on fitness, given that this species routinely experiences such temperature fluctuations in nature. Our results may therefore explain the low densities of *D. birchii* found at these low elevation sites in the field (Bridle et al., 2009; O'Brien et al., 2017), despite productivity in the laboratory being higher at similar constant temperatures. This study, combined with the findings of Zeh et al. (Zeh et al., 2012; Zeh et al., 2014), demonstrates that sub-lethal effects of increased climatic variation on male fitness are prevalent across even distantly-related tropical ectotherms. Moreover, given evidence that that upper thermal limits of ectotherms are more constrained across latitudinal scales than lower limits (Overgaard et al., 2014), the sensitivity of male reproductive success to even slightly increased temperature fluctuations suggests that increasing thermal variability could have strongly detrimental impacts on population persistence across both tropical and temperate ectotherms.

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Figure legends

Figure 1. Performance of males following development in 24°C constant and fluctuating treatments (Experiment I, left) and 17°C constant and fluctuating treatments (Experiment II, right). The black line shows median values, boxes showing interquartile range with whiskers, outliers shown as circles for: (i) total number of offspring (ii) number of offspring produced per mating (iii) wing centroid size (in pixels). F-ratio, degrees of freedom (in parentheses) and associated probability (p) are shown for effect of treatment (also see Table SM1).

Figure 2. Performance of males after development in constant 21°C, fluctuating 24°C and constant 27°C conditions (Experiment III). Black line shows median values, boxes showing interquartile range with whiskers, outliers shown as circles for: total offspring per male (left), wing centroid size (right). P-values for Tukey's HSD *post hoc* test significance (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).

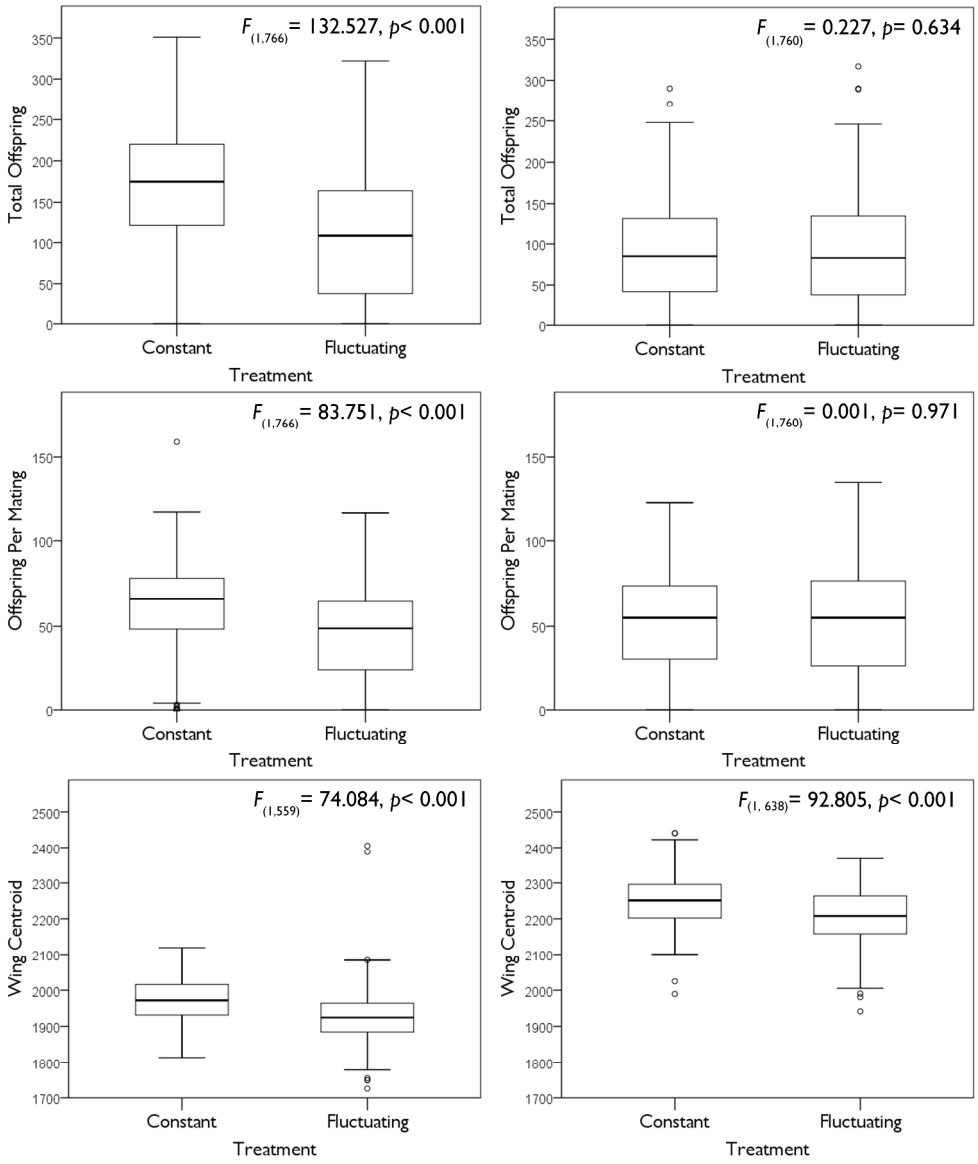

Figure 1. Performance of males following development in 24°C constant and fluctuating treatments (Experiment I, left) and 17°C constant and fluctuating treatments (Experiment II, right). The black line shows median values, boxes showing interquartile range with whiskers, outliers shown as circles for: (i) total number of offspring (ii) number of offspring produced per mating (iii) wing centroid size (in pixels). *F*-ratio, degrees of freedom (in parentheses) and associated probability (*p*) are shown for effect of treatment (also see Table SM1).

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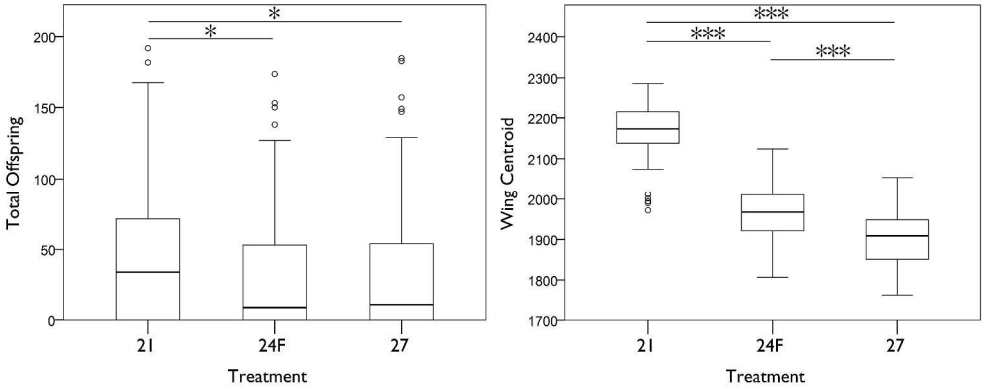

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1595x648mm (96 x 96 DPI)

Supplementary material

Materials and Methods

Origin of isofemale lines

Flies were collected at each site using banana baited buckets, sampled daily using fine sweep nets and sorted under a microscope using light CO_2 anaesthesia to isolate *D. birchii* females. Field-mated females were placed individually in vials to lay, to found isofemale lines. Each line was maintained across 3–4 40 ml vials containing 10 ml of standard *Drosophila* food medium (agar, potato, raw sugar, inactive yeast, propionic acid, nipagin supplemented with live yeast) at populations of ~100 individuals per generation for each line.

Mass-bred stocks were reared in 400 ml bottles with 100 ml of *Drosophila* medium. All lines and the mass-bred population were maintained at 19°C on a 12:12-h light:dark cycle at 60% relative humidity (RH) prior to the experiments.

Temperature data for Mount Lewis transect

Elevation	Site	Mean Daily Min	Mean Daily Mean	Mean Daily Max	Mean - Min	Max - Mean
Low	L1	21.57	24.09	27.68	2.52	3.59
Low	L2	21.85	24.10	27.44	2.25	3.35
High	H1	17.19	18.42	20.02	1.23	1.60
High	H2	17.10	18.30	19.86	1.21	1.57

Figure SM1. *Showing the daily high, low and mean temperature (in °C) for 2 high- and 2 low- elevation sites on Mount Lewis, from dataloggers collecting hourly temperature data from February 2010 to June 2012.*

Fluctuating regime cycle

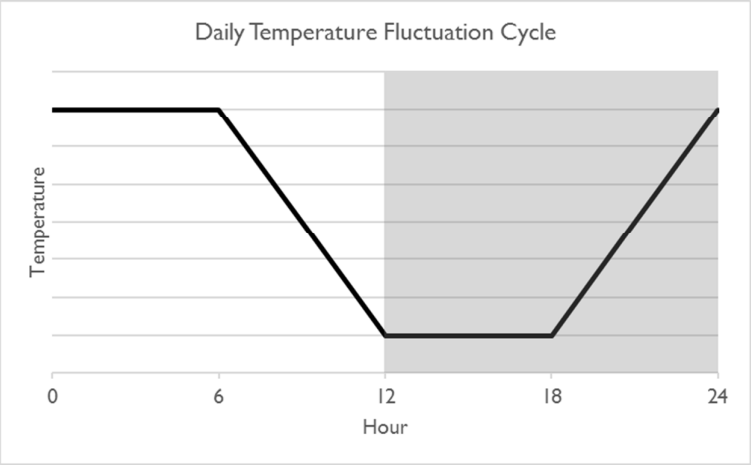

Figure SM2*. Showing daily fluctuating regime. White area represents 12-h light period, grey represents 12-h dark period. The temperatures were constant for 6-h in each period and then ramped either up or down for the next 6-h. High temperatures occurred during the light period and lows during the night period mimicking a natural diurnal cycle.*

Morphometric analysis of wing centroid size

Right wings from preserved males from each of the 20 lines for each temperature treatment were mounted on microscope slides with coverslips affixed with Aquatex mounting medium (Merck). Each wing was photographed using a Nikon SMZ800 microscope with GXCAM-9 camera attachment (GT vision) and subsequently landmarked at 10 wing vein intersections using tps (Util32 v.1.74 , DIG2w32 v.2.29 and Relw32 v.1.67) software by F.J. Rohlf (2015).

Statistical analyses: calculation of H^2

H^2 was calculated following the method of Hoffmann & Parsons (1988)(see references in main manuscript) as:

$$H^2 = \frac{1}{2F_t} V_b/(V_b + V_w) \qquad (1)$$

where F_t is the inbreeding coefficient after t generations as isofemale lines, calculated according to Falconer & Mackay (1996) as:

$$F_t = \frac{1}{2N} + \left(1 - \frac{1}{2N}\right) F_{t-1} \quad (2)$$

where N is the population size in each generation. We calculated F_t assuming a population size of 100 in each generation after establishment, and 75 generations as isofemale lines. We assumed that offspring in the first generation were all full-sibs (*i.e.* $N_{Gen0} = 2$; $F_0 = 0.25$).

We evaluated whether H^2 was significantly different from zero by comparing models with and without the random effect of line, using a likelihood ratio test. We calculated 95% confidence intervals (CI) on variance components (V_b and V_w) using bootstrap resampling with 1000 simulations, implemented using the *confint* function in *lme*4. We used these to calculate lower and upper 95% CIs around H^2 estimates using the same formula as for H^2. We considered H^2 estimates for a trait across treatments to be significantly different if there was no overlap in 95% CIs.

Results

Experiment	*Trait*	*F*	*df*	*p*
Experiment I: Mean 24°C	Total offspring	132.527	1,766	<0.001
	Offspring per mating	83.751	1,766	<0.001
	Wing size	74.084	1,559	<0.001
Experiment II: Mean 17°C	Total offspring	0.227	1,760	0.634
	Offspring per mating	0.001	1,760	0.971
	Wing size	92.805	1,638	<0.001

Table SM1. Results of ANOVA testing for effect of temperature treatment (constant vs fluctuating) on total productivity, offspring per mating and wing size in Experiments I and II. Treatment was a fixed factor in models that included isofemale line nested within transect as random effects (altitude of origin did not improve model fit and was therefore dropped from the model- see Methods). *F*-ratio, degrees of freedom (*df*) and associated probability (*p*) are shown for measured traits in Experiment I (top) and Experiment II (bottom). The fluctuating temperature treatment reduced total offspring, mean offspring per mating and body size of males in Experiment I, and body size of males in Experiment II (see also Fig. 1).

	Variance component	***Total offspring***			***Offspring per mating***			***Wing Size***		
		Variance	*%*	*p*	*Variance*	*%*	*p*	*Variance*	*%*	*p*
Experiment I	Line (Altitude (Transect))	904.03	13.59	$<2.2 \times 10^{-16}$	120.7	14.87	$<2.2 \times 10^{-16}$	1445.7	27.19	$<2.2 \times 10^{-16}$
	Altitude (Transect)	0.0	0	1	0.0	0	1	0	0	1
	Transect	20.58	0.31	1	0.0	0	1	439.8	8.27	0.5066
	Residual	5729.29	86.10	-	691.1	85.13	-	3432.1	64.54	-
Experiment II	Line (Altitude (Transect))	768.3	19.33	$<2.2 \times 10^{-16}$	175.4	17.99	$<2.2 \times 10^{-16}$	2160.0	35.24	$<2.2 \times 10^{-16}$
	Altitude (Transect)	1.083×10^{-11}	<0.01	1	1.169×10^{-11}	<0.01	1	0	0	1
	Transect	3.418×10^{-11}	<0.01	1	2.516×10^{-10}	<0.01	1	485.9	7.93	0.6507
	Residual	3207	80.68	-	799.8	82.01	-	3483.2	56.83	-

Table SM2. Results of model comparisons for variance components (random effects). Nesting of each component in the model shown by sequential parentheses. Showing variance, proportion of variance (%) with residual. P-values derived from ANOVAs between models where individual variance components are excluded and compared to the full model to assess contribution to the model fit.

Experiment	*Constant*		*Fluctuating*	
	Mean	*SD*	*Mean*	*SD*
Experiment I: Mean 24°C	5.28	8.90	17.59	18.32
Experiment II: Mean 17°C	10.08	11.51	15.87	15.87

Table SM3. Mean proportion (%) of males per line having no offspring in Experiment I/II treatments with standard deviation. Independent samples T-test show that differences between proportions are significant for Experiment I: t= -2.701(38), p= **0.01** but non-significant for Experiment II: t= -1.155(38), p= 0.255.

Treatment	*Comparison*	*Total Offspring*	*Wing Size*
21°C Constant	24F	**0.024**	**<0.001**
	27C	**0.043**	**<0.001**
24°C Fluctuating	21C	**0.024**	**<0.001**
	27C	0.977	**<0.001**
27°C Constant	21C	**0.043**	**<0.001**
	24F	0.977	**<0.001**

Table SM4. Probability (p) for Tukey's HSD *post hoc* tests for Experiment III. Comparing mean total offspring per male and wing size traits under 3 temperature treatments: 21°C constant, 24°C fluctuating and 27°C constant.

Trait 1	*Trait 2*	*Treatment*			
		Mean 24°C		*Mean 17°C*	
		Constant	*Fluctuating*	*Constant*	*Fluctuating*
Total offspring	Offspring per mating	**0.934** (p = 3 x 10^{-12})	**0.909** (p = 5.2 x 10^{-11})	**0.872** (p = 1.14 x 10^{-9})	**0.777** (p = 1.74 x 10^{-7})
Total offspring	Wing size	0.002 (p = 0.322)	0.046 (p = 0.183)	**0.207** (p = 0.025)	0.073 (p = 0.131)
Offspring per mating	Wing size	0.001 (p = 0.326)	0.105 (p = 0.089)	**0.169** (p = 0.041)	0.00 (p = 0.476)

Table SM5. Genetic correlations (r_G) between each pair of traits of males, estimated from the trait correlations of the isofemale line means within each rearing environment. Numbers in parentheses are probabilities that r_G = 0. Correlations where r_G is significantly greater than 0 (corrected for multiple comparisons) are in **bold**.

R code:

```
install.packages("lme4")
install.packages("LMERConvenienceFunctions")


#Experiment 1
Data_24 <- Experiment1_24deg_data


#Total offspring
Exp1_model1.0 <- lmer(Cumulative_offspring ~ Treatment + 1|Transect/Altitude/Line, data = Data_24)
summary(Exp1_model1.0)
anova(Exp1_model1.0)
Exp1_model1.1 <- lmer(Cumulative_offspring ~ 1 + 1|Transect/Altitude/Line, data = Data_24)#Run a model with treatment excluded
anova(Exp1_model1.0, Exp1_model1.1)#Compare the full model with a model excluding treatment to evaluate the effect of treatment on this measure of fitness
Exp1_model1.2 <- lmer(Cumulative_offspring ~ Treatment + 1|Transect/Altitude, data = Data_24) #Run a model excluding the line effect
anova(Exp1_model1.0, Exp1_model1.2)#compare models to evaluate significance of variance due to line
Exp1_model1.3 <- lmer(Cumulative_offspring ~ Treatment + 1|Transect/Line, data = Data_24) #Run a model excluding the altitude effect
anova(Exp1_model1.0, Exp1_model1.3)#compare models to evaluate significance of variance due to altitude within transect
Exp1_model1.4 <- lmer(Cumulative_offspring ~ Treatment + 1|Line, data = Data_24) #Run a model excluding the transect effect
anova(Exp1_model1.3, Exp1_model1.4)#compare models to evaluate significance of variance due to transect

summary(Exp1_model1.3)


#Offspring per mating
Exp1_model3.0 <- lmer(Offspring_per_mating ~ Treatment + 1|Transect/Altitude/Line, data = Data_24)
summary(Exp1_model3.0)
Exp1_model3.1 <- lmer(Offspring_per_mating ~ 1 + 1|Transect/Altitude/Line, data = Data_24)
anova(Exp1_model3.0, Exp1_model3.1)
Exp1_model3.2 <- lmer(Offspring_per_mating ~ Treatment + 1|Transect/Altitude, data = Data_24)
anova(Exp1_model3.2, Exp1_model3.0)
Exp1_model3.3 <- lmer(Offspring_per_mating ~ Treatment + 1|Transect/Line, data = Data_24)
anova(Exp1_model3.3, Exp1_model3.0)
Exp1_model3.4 <- lmer(Offspring_per_mating ~ Treatment + 1|Line, data = Data_24)
anova(Exp1_model3.3, Exp1_model3.4)

summary(Exp1_model3.3)


#Body size
Wings_24 <- Wing_centroid_24_Experiment1


Exp1_model4.0 <- lmer(Wing_centroid ~ Treatment + 1|Transect/Altitude/Line, data = Wings_24)
summary(Exp1_model4.0)
Exp1_model4.1 <- lmer(Wing_centroid ~ 1 + 1|Transect/Altitude/Line, data = Wings_24)
anova(Exp1_model4.0, Exp1_model4.1)
```

```
Exp1_model4.2 <- lmer(Wing_centroid ~ Treatment + 1|Transect/Altitude, data = Wings_24)
anova(Exp1_model4.0, Exp1_model4.2)
Exp1_model4.3 <- lmer(Wing_centroid ~ Treatment + 1|Transect/Line, data = Wings_24)
anova(Exp1_model4.0, Exp1_model4.3)
Exp1_model4.4 <- lmer(Wing_centroid ~ Treatment + 1|Line, data = Wings_24)
anova(Exp1_model4.4, Exp1_model4.3)

summary(Exp1_model4.3)


#Look at among-line variance separately for the two treatments

Data_24_constant <- subset(Data_24, Treatment=="Constant")
Data_24_fluctuating <- subset(Data_24, Treatment == "Fluctuating")

#Total offspring
#24 degree constant

Exp1_model1.1a <- lmer(Cumulative_offspring ~ 1 + 1|Transect/Altitude/Line, data = Data_24_constant)
Exp1_model1.2a <- lmer(Cumulative_offspring ~ 1 + 1|Transect/Altitude, data = Data_24_constant)
anova(Exp1_model1.1a, Exp1_model1.2a)Exp1_model1.3a <- lmer(Cumulative_offspring ~ 1 +
1|Transect/Line, data = Data_24_constant)
anova(Exp1_model1.1a, Exp1_model1.3a)
Exp1_model1.4a <- lmer(Cumulative_offspring ~ 1 + 1|Line, data = Data_24_constant)
anova(Exp1_model1.3a, Exp1_model1.4a) summary(Exp1_model1.4a) #Estimate 95% confidence intervals
using bootstrapping
Warm_constant_TotOffspring_ci <- confint(Exp1_model1.4a, parm="theta_", level=0.95, method=c("boot"),
        nsim =1000,
        boot.type=c("perc", "basic", "norm"), quiet = TRUE, oldNames=FALSE)
#gives 95% CIs on SDs, which can be squared to get the variance

#24 degree fluctuating
Exp1_model1.1b <- lmer(Cumulative_offspring ~ 1 + 1|Transect/Altitude/Line, data = Data_24_fluctuating)
Exp1_model1.2b <- lmer(Cumulative_offspring ~ 1 + 1|Transect/Altitude, data = Data_24_fluctuating)
anova(Exp1_model1.1b, Exp1_model1.2b)
Exp1_model1.3b <- lmer(Cumulative_offspring ~ 1 + 1|Transect/Line, data = Data_24_fluctuating)
anova(Exp1_model1.1b, Exp1_model1.3b)
Exp1_model1.4b <- lmer(Cumulative_offspring ~ 1 + 1|Line, data = Data_24_fluctuating)
anova(Exp1_model1.3b, Exp1_model1.4b) summary(Exp1_model1.4b)
Warm_fluctuating_TotOffspring_ci <- confint(Exp1_model1.4b1, parm="theta_", level=0.95,
method=c("boot"),
                nsim =1000,
                boot.type=c("perc", "basic", "norm"), quiet = TRUE, oldNames=FALSE)
#Offspring per mating
#24 degree constant

Exp1_model3.1a <- lmer(Offspring_per_mating ~ 1 + 1|Transect/Altitude/Line, data = Data_24_constant)
Exp1_model3.2a <- lmer(Offspring_per_mating ~ 1 + 1|Transect/Altitude, data = Data_24_constant)
anova(Exp1_model3.1a, Exp1_model3.2a)
Exp1_model3.3a <- lmer(Offspring_per_mating ~ 1 + 1|Transect/Line, data = Data_24_constant)
anova(Exp1_model3.1a, Exp1_model3.3a)

Exp1_model3.4a <- lmer(Offspring_per_mating ~ 1 + 1|Line, data = Data_24_constant)
anova(Exp1_model3.3a, Exp1_model3.4a)
summary(Exp1_model3.4a)
Warm_constant_OffspringPerMating_ci <- confint(Exp1_model3.4a1, parm="theta_", level=0.95,
method=c("boot"),
```

```
                    nsim =1000,
                    boot.type=c("perc", "basic", "norm"), quiet = TRUE, oldNames=FALSE)

#24 degree fluctuating

Exp1_model3.1b <- lmer(Offspring_per_mating ~ 1 + 1|Transect/Altitude/Line, data = Data_24_fluctuating)
Exp1_model3.2b <- lmer(Offspring_per_mating ~ 1 + 1|Transect/Altitude, data = Data_24_fluctuating)
anova(Exp1_model3.1b, Exp1_model3.2b)
Exp1_model3.3b <- lmer(Offspring_per_mating ~ 1 + 1|Transect/Line, data = Data_24_fluctuating)
anova(Exp1_model3.1b, Exp1_model3.3b)
Exp1_model3.4b <- lmer(Offspring_per_mating ~ 1 + 1|Line, data = Data_24_fluctuating)
anova(Exp1_model3.3b, Exp1_model3.4b)
summary(Exp1_model3.4b) Warm_fluctuating_OffspringPerMating_ci <- confint(Exp1_model3.4b1,
parm="theta_", level=0.95, method=c("boot"),
                    nsim =1000,
                    boot.type=c("perc", "basic", "norm"), quiet = TRUE, oldNames=FALSE)

#Body size

Wings_24_constant <- subset(Wings_24, Treatment=="Constant")
Wings_24_fluctuating <- subset(Wings_24, Treatment == "Fluctuating")

#24 degree constant

Exp1_model4.1a <- lmer(Wing_centroid ~ 1 + 1|Transect/Altitude/Line, data = Wings_24_constant)
Exp1_model4.2a <- lmer(Wing_centroid ~ 1 + 1|Transect/Altitude, data = Wings_24_constant)
anova(Exp1_model4.1a, Exp1_model4.2a)
Exp1_model4.3a <- lmer(Wing_centroid ~ 1 + 1|Transect/Line, data = Wings_24_constant)
anova(Exp1_model4.1a, Exp1_model4.3a)
Exp1_model4.4a <- lmer(Wing_centroid ~ 1 + 1|Line, data = Wings_24_constant)
anova(Exp1_model4.4a, Exp1_model4.3a)
summary(Exp1_model4.4a)
Warm_constant_wings_ci <- confint(Exp1_model4.4a, parm="theta_", level=0.95, method=c("boot"),
                    nsim =1000,
                    boot.type=c("perc", "basic", "norm"), quiet = TRUE, oldNames=FALSE)

#24 degree fluctuating

Exp1_model4.1b <- lmer(Wing_centroid ~ 1 + 1|Transect/Altitude/Line, data = Wings_24_fluctuating)
Exp1_model4.2b <- lmer(Wing_centroid ~ 1 + 1|Transect/Altitude, data = Wings_24_fluctuating)
anova(Exp1_model4.1b, Exp1_model4.2b)
Exp1_model4.3b <- lmer(Wing_centroid ~ 1 + 1|Transect/Line, data = Wings_24_fluctuating)
anova(Exp1_model4.3b, Exp1_model4.1b)
Exp1_model4.4b <- lmer(Wing_centroid ~ 1 + 1|Line, data = Wings_24_fluctuating)
anova(Exp1_model4.4b, Exp1_model4.3b)
summary(Exp1_model4.4b)
Warm_fluctuating_wings_ci <- confint(Exp1_model4.4b, parm="theta_", level=0.95, method=c("boot"),
                    nsim =1000,
                    boot.type=c("perc", "basic", "norm"), quiet = TRUE, oldNames=FALSE)
```

#Experiment 2

```
Data_17 <- Experiment2_17deg_data1
```

```
#Total offspring

Exp2_model1.0 <- lmer(Cumulative_Offspring ~ Treatment + 1|Transect/Altitude/Line, data = Data_17)
summary(Exp2_model1.0)
Exp2_model1.1 <- lmer(Cumulative_Offspring ~ 1 + 1|Transect/Altitude/Line, data = Data_17)
anova(Exp2_model1.0, Exp2_model1.1) Exp2_model1.2 <- lmer(Cumulative_Offspring ~ 1 +
1|Transect/Altitude, data = Data_17)
anova(Exp2_model1.1, Exp2_model1.2)
Exp2_model1.3 <- lmer(Cumulative_Offspring ~ 1 + 1|Transect/Line, data = Data_17)
anova(Exp2_model1.1, Exp2_model1.3)
Exp2_model1.4 <- lmer(Cumulative_Offspring ~ 1 + 1|Line, data = Data_17)  anova(Exp2_model1.3,
Exp2_model1.4) summary(Exp2_model1.4)


#Offspring_per_mating

Exp2_model3.0 <- lmer(Offspring_per_mating ~ Treatment + 1|Transect/Altitude/Line, data = Data_17)
summary(Exp2_model3.0)
Exp2_model3.1 <- lmer(Offspring_per_mating ~ 1 + 1|Transect/Altitude/Line, data = Data_17)
anova(Exp2_model3.0, Exp2_model3.1)
Exp2_model3.2 <- lmer(Offspring_per_mating ~ 1 + 1|Transect/Altitude, data = Data_17)
anova(Exp2_model3.1, Exp2_model3.2)
Exp2_model3.3 <- lmer(Offspring_per_mating ~ 1 + 1|Transect/Line, data = Data_17)  anova(Exp2_model3.1,
Exp2_model3.3) Exp2_model3.4 <- lmer(Offspring_per_mating ~ 1 + 1|Line, data = Data_17)
anova(Exp2_model3.3, Exp2_model3.4) summary(Exp2_model3.4)



#Body size

Wings_17 <- Wing_centroid_17_Experiment2


Exp2_model4.0 <- lmer(Wing_centroid ~ Treatment + 1|Transect/Altitude/Line, data = Wings_17)
summary(Exp2_model4.0)
Exp2_model4.1 <- lmer(Wing_centroid ~ 1 + 1|Transect/Altitude/Line, data = Wings_17)
anova(Exp2_model4.0, Exp2_model4.1)
Exp2_model4.2 <- lmer(Wing_centroid ~ Treatment + 1|Transect/Altitude, data = Wings_17)
anova(Exp2_model4.0, Exp2_model4.2)
Exp2_model4.3 <- lmer(Wing_centroid ~ Treatment + 1|Transect/Line, data = Wings_17)
anova(Exp2_model4.0, Exp2_model4.3)
Exp2_model4.4 <- lmer(Wing_centroid ~ Treatment + 1|Line, data = Wings_17)
anova(Exp2_model4.4, Exp2_model4.3)
summary(Exp1_model4.4)



#Look at among-line variance separately for the two treatments

Data_17_constant <- subset(Data_17, Treatment=="Constant")
Data_17_fluctuating <- subset(Data_17, Treatment=="Fluctuating")

#Total offspring

#17 degree constant

Exp2_model1.1a <- lmer(Cumulative_Offspring ~ 1 + 1|Transect/Altitude/Line, data = Data_17_constant)
```

```
Exp2_model1.2a <- lmer(Cumulative_Offspring ~ 1 + 1|Transect/Altitude, data = Data_17_constant)
anova(Exp2_model1.1a, Exp2_model1.2a)
Exp2_model1.3a <- lmer(Cumulative_Offspring ~ 1 + 1|Transect/Line, data = Data_17_constant)
anova(Exp2_model1.1a, Exp2_model1.3a)
Exp2_model1.4a <- lmer(Cumulative_Offspring ~ 1 + 1|Line, data = Data_17_constant)
anova(Exp2_model1.3a, Exp2_model1.4a)
summary(Exp2_model1.4a)
Cool_constant_TotOffspring_ci <- confint(Exp2_model1.4a, parm="theta_", level=0.95, method=c("boot"),
                        nsim =1000,
                        boot.type=c("perc", "basic", "norm"), quiet = TRUE, oldNames=FALSE)

#17 degree fluctuating

Exp2_model1.1b <- lmer(Cumulative_Offspring ~ 1 + 1|Transect/Altitude/Line, data = Data_17_fluctuating)
Exp2_model1.2b <- lmer(Cumulative_Offspring ~ 1 + 1|Transect/Altitude, data = Data_17_fluctuating)
anova(Exp2_model1.1b, Exp2_model1.2b)
Exp2_model1.3b <- lmer(Cumulative_Offspring ~ 1 + 1|Transect/Line, data = Data_17_fluctuating)
anova(Exp2_model1.3b, Exp2_model1.1b)
Exp2_model1.4b <- lmer(Cumulative_Offspring ~ 1 + 1|Line, data = Data_17_fluctuating)
anova(Exp2_model1.3b, Exp2_model1.4b)

summary(Exp2_model1.4b)
Cool_fluctuating_TotOffspring_ci <- confint(Exp2_model1.4b, parm="theta_", level=0.95, method=c("boot"),
                        nsim =1000,
                        boot.type=c("perc", "basic", "norm"), quiet = TRUE, oldNames=FALSE)


#Offspring per mating

#17 degree constant

Exp2_model3.1a <- lmer(Offspring_per_mating ~ 1 + 1|Transect/Altitude/Line, data = Data_17_constant)
Exp2_model3.2a <- lmer(Offspring_per_mating ~ 1 + 1|Transect/Altitude, data = Data_17_constant)
anova(Exp2_model3.1a, Exp2_model3.2a)
Exp2_model3.3a <- lmer(Offspring_per_mating ~ 1 + 1|Transect/Line, data = Data_17_constant)
anova(Exp2_model3.3a, Exp2_model3.1a)
Exp2_model3.4a <- lmer(Offspring_per_mating ~ 1 + 1|Line, data = Data_17_constant)
anova(Exp2_model3.3a, Exp2_model3.4a)

summary(Exp2_model3.4a)
Cool_constant_OffspringPerMating_ci <- confint(Exp2_model3.4a, parm="theta_", level=0.95,
method=c("boot"),
                        nsim =1000,
                        boot.type=c("perc", "basic", "norm"), quiet = TRUE, oldNames=FALSE)


#17 degree fluctuating

Exp2_model3.1b <- lmer(Offspring_per_mating ~ 1 + 1|Transect/Altitude/Line, data = Data_17_fluctuating)
Exp2_model3.2b <- lmer(Offspring_per_mating ~ 1 + 1|Transect/Altitude, data = Data_17_fluctuating)
anova(Exp2_model3.1b, Exp2_model3.2b)
Exp2_model3.3b <- lmer(Offspring_per_mating ~ 1 + 1|Transect/Line, data = Data_17_fluctuating)
anova(Exp2_model3.3b, Exp2_model3.1b)
Exp2_model3.4b <- lmer(Offspring_per_mating ~ 1 + 1|Line, data = Data_17_fluctuating)
anova(Exp2_model3.3b, Exp2_model3.4b)

summary(Exp2_model3.4b)
```

```
Cool_fluctuating_OffspringPerMating_ci <- confint(Exp2_model3.4b, parm="theta_", level=0.95,
method=c("boot"),
                              nsim =1000,
                              boot.type=c("perc", "basic", "norm"), quiet = TRUE, oldNames=FALSE)



#Body size

Wings_17_constant <- subset(Wings_17, Treatment=="Constant")
Wings_17_fluctuating <- subset(Wings_17, Treatment == "Fluctuating")

#17 degree constant

Exp2_model4.1a <- lmer(Wing_centroid ~ 1 + 1|Transect/Altitude/Line, data = Wings_17_constant)
Exp2_model4.2a <- lmer(Wing_centroid ~ 1 + 1|Transect/Altitude, data = Wings_17_constant)
anova(Exp2_model4.1a, Exp2_model4.2a)
Exp2_model4.3a <- lmer(Wing_centroid ~ 1 + 1|Transect/Line, data = Wings_17_constant)
anova(Exp2_model4.1a, Exp2_model4.3a)
Exp2_model4.4a <- lmer(Wing_centroid ~ 1 + 1|Line, data = Wings_17_constant)
anova(Exp2_model4.4a, Exp2_model4.3a) summary(Exp2_model4.4a)
Cool_constant_wings_ci <- confint(Exp2_model4.4a, parm="theta_", level=0.95, method=c("boot"),
                              nsim =1000,
                              boot.type=c("perc", "basic", "norm"), quiet = TRUE, oldNames=FALSE)



#17 degree fluctuating

Exp2_model4.1b <- lmer(Wing_centroid ~ 1 + 1|Transect/Altitude/Line, data = Wings_17_fluctuating)
Exp2_model4.2b <- lmer(Wing_centroid ~ 1 + 1|Transect/Altitude, data = Wings_17_fluctuating)
anova(Exp2_model4.1b, Exp2_model4.2b)
Exp2_model4.3b <- lmer(Wing_centroid ~ 1 + 1|Transect/Line, data = Wings_17_fluctuating)
anova(Exp2_model4.3b, Exp2_model4.1b)
Exp2_model4.4b <- lmer(Wing_centroid ~ 1 + 1|Line, data = Wings_17_fluctuating)
anova(Exp2_model4.4b, Exp2_model4.3b)
summary(Exp2_model4.4b)
Cool_fluctuating_wings_ci <- confint(Exp2_model4.4b, parm="theta_", level=0.95, method=c("boot"),
                        nsim =1000,
                        boot.type=c("perc", "basic", "norm"), quiet = TRUE, oldNames=FALSE)
```

#Experiment 3

```
#Total offspring

Exp3_model1.0 <- lmer(Total_offspring ~ Treatment + 1|Transect/Line, data = Experiment3_data)
summary(Exp3_model1.0)
Exp3_model1.1 <- lmer(Total_offspring ~ 1 + 1|Transect/Line, data = Experiment3_data)
anova(Exp3_model1.0, Exp3_model1.1)
Exp3_model1.2 <- lmer(Total_offspring ~ Treatment + 1|Transect, data = Experiment3_data)
anova(Exp3_model1.0, Exp3_model1.2)
Exp3_model1.3 <- lmer(Total_offspring ~ Treatment + 1|Line, data = Experiment3_data)
anova(Exp3_model1.0, Exp3_model1.3)
```

```
#Offspring per mating

Exp3_model3.0 <- lmer(Offspring_per_mating ~ Treatment + 1|Transect/Line, data = Experiment3_data)
summary(Exp3_model3.0)
Exp3_model3.1 <- lmer(Offspring_per_mating ~ 1 + 1|Transect/Line, data = Experiment3_data)
anova(Exp3_model3.0, Exp3_model3.1)
Exp3_model3.2 <- lmer(Offspring_per_mating ~ 1 + 1|Transect, data = Experiment3_data)
anova(Exp3_model3.1, Exp3_model3.2)
Exp3_model3.3 <- lmer(Offspring_per_mating ~ 1 + 1|Line, data = Experiment3_data)
anova(Exp3_model3.1, Exp3_model3.3)
```

#Trait correlations based on line means in experiments 1 and 2

```
#Correlation of pairs of traits within each rearing environment (treatment)

Data_24_constant_linemeans <- subset(Data_24_linemeans, Treatment=="Constant")
Data_24_fluctuating_linemeans <- subset(Data_24_linemeans, Treatment=="Fluctuating")
Data_17_constant_linemeans <- subset(Data_17_linemeans, Treatment=="Constant")
Data_17_fluctuating_linemeans <- subset(Data_17_linemeans, Treatment=="Fluctuating")

#Total offspring vs Offspring per mating

#24 constant
Corr_TotalOffspring_OffspringPerMating_24Constant <- lm(Mean_offspring_per_mating ~
Mean_total_offspring, data = Data_24_constant_linemeans)
summary(Corr_TotalOffspring_OffspringPerMating_24Constant)

#24 fluctuating
Corr_TotalOffspring_OffspringPerMating_24Fluctuating <- lm(Mean_offspring_per_mating ~
Mean_total_offspring, data = Data_24_fluctuating_linemeans)
summary(Corr_TotalOffspring_OffspringPerMating_24Fluctuating)

#17 constant
Corr_TotalOffspring_OffspringPerMating_17Constant <- lm(Mean_offspring_per_mating ~
Mean_total_offspring, data = Data_17_constant_linemeans)
summary(Corr_TotalOffspring_OffspringPerMating_17Constant)

#17 fluctuating
Corr_TotalOffspring_OffspringPerMating_17Fluctuating <- lm(Mean_offspring_per_mating ~
Mean_total_offspring, data = Data_17_fluctuating_linemeans)
summary(Corr_TotalOffspring_OffspringPerMating_17Fluctuating)

#Total offspring vs Body size

#24 constant
Corr_TotalOffspring_WingSize_24Constant <- lm(Mean_wing_centroid ~ Mean_total_offspring, data =
Data_24_constant_linemeans)
summary(Corr_TotalOffspring_WingSize_24Constant)

#24 fluctuating
Corr_TotalOffspring_WingSize_24Fluctuating <- lm(Mean_wing_centroid ~ Mean_total_offspring, data =
Data_24_fluctuating_linemeans)
summary(Corr_TotalOffspring_WingSize_24Fluctuating)
```

```
#17 constant
Corr_TotalOffspring_WingSize_17Constant <- lm(Mean_wing_centroid ~ Mean_total_offspring, data =
Data_17_constant_linemeans)
summary(Corr_TotalOffspring_WingSize_17Constant)

#17 fluctuating
Corr_TotalOffspring_WingSize_17Fluctuating <- lm(Mean_wing_centroid ~ Mean_total_offspring, data =
Data_17_fluctuating_linemeans)
summary(Corr_TotalOffspring_WingSize_17Fluctuating)


#Offspring per mating vs Body size

#24 constant
Corr_OffspringPerMating_WingSize_24Constant <- lm(Mean_wing_centroid ~ Mean_offspring_per_mating,
data = Data_24_constant_linemeans)
summary(Corr_OffspringPerMating_WingSize_24Constant)

#24 fluctuating
Corr_OffspringPerMating_WingSize_24Fluctuating <- lm(Mean_wing_centroid ~ Mean_offspring_per_mating,
data = Data_24_fluctuating_linemeans)
summary(Corr_OffspringPerMating_WingSize_24Fluctuating)

#17 constant
Corr_OffspringPerMating_WingSize_17Constant <- lm(Mean_wing_centroid ~ Mean_offspring_per_mating,
data = Data_17_constant_linemeans)
summary(Corr_OffspringPerMating_WingSize_17Constant)

#17 fluctuating
Corr_OffspringPerMating_WingSize_17Fluctuating <- lm(Mean_wing_centroid ~ Mean_offspring_per_mating,
data = Data_17_fluctuating_linemeans)
summary(Corr_OffspringPerMating_WingSize_17Fluctuating)


#Cross-environment correlations of each trait (same trait in different rearing environments) in experiments
1 and 2

#Total offspring

#24 constant vs 24 fluctuating
Corr_TotalOffspring_24Constant_24Fluctuating <- lm(Mean_total_24constant ~ Mean_total_24fluctuating,
data = All_linemeans)
summary(Corr_TotalOffspring_24Constant_24Fluctuating)

#24 constant vs 17 constant
Corr_TotalOffspring_24Constant_17Constant <- lm(Mean_total_24constant ~ Mean_total_17constant, data =
All_linemeans)
summary(Corr_TotalOffspring_24Constant_17Constant)

#24 constant vs 17 fluctuating
Corr_TotalOffspring_24Constant_17Fluctuating <- lm(Mean_total_24constant ~ Mean_total_17fluctuating,
data = All_linemeans)
summary(Corr_TotalOffspring_24Constant_17Fluctuating)

#24 fluctuating vs 17 constant
```

```
Corr_TotalOffspring_24Fluctuating_17Constant <- lm(Mean_total_24fluctuating ~ Mean_total_17constant,
data = All_linemeans)
summary(Corr_TotalOffspring_24Fluctuating_17Constant)

#24 fluctuating vs 17 fluctuating
Corr_TotalOffspring_24Fluctuating_17Fluctuating <- lm(Mean_total_24fluctuating ~
Mean_total_17fluctuating, data = All_linemeans)
summary(Corr_TotalOffspring_24Fluctuating_17Fluctuating)

#17 constant vs 17 fluctuating
Corr_TotalOffspring_17Constant_17Fluctuating <- lm(Mean_total_17constant ~ Mean_total_17fluctuating,
data = All_linemeans)
summary(Corr_TotalOffspring_17Constant_17Fluctuating)


#Offspring per mating

#24 constant vs 24 fluctuating
Corr_OffspringPerMating_24Constant_24Fluctuating <- lm(Mean_OffspringPerMating_24constant ~
Mean_OffspringPerMating_24fluctuating, data = All_linemeans)
summary(Corr_OffspringPerMating_24Constant_24Fluctuating)

#24 constant vs 17 constant
Corr_OffspringPerMating_24Constant_17Constant <- lm(Mean_OffspringPerMating_24constant ~
Mean_OffspringPerMating_17constant, data = All_linemeans)
summary(Corr_OffspringPerMating_24Constant_17Constant)

#24 constant vs 17 fluctuating
Corr_OffspringPerMating_24Constant_17Fluctuating <- lm(Mean_OffspringPerMating_24constant ~
Mean_OffspringPerMating_17fluctuating, data = All_linemeans)
summary(Corr_OffspringPerMating_24Constant_17Fluctuating)

#24 fluctuating vs 17 constant
Corr_OffspringPerMating_24Fluctuating_17Constant <- lm(Mean_OffspringPerMating_24fluctuating ~
Mean_OffspringPerMating_17constant, data = All_linemeans)
summary(Corr_OffspringPerMating_24Fluctuating_17Constant)

#24 fluctuating vs 17 fluctuating
Corr_OffspringPerMating_24Fluctuating_17Fluctuating <- lm(Mean_OffspringPerMating_24fluctuating ~
Mean_OffspringPerMating_17fluctuating, data = All_linemeans)
summary(Corr_OffspringPerMating_24Fluctuating_17Fluctuating)

#17 constant vs 17 fluctuating
Corr_OffspringPerMating_17Constant_17Fluctuating <- lm(Mean_OffspringPerMating_17constant ~
Mean_OffspringPerMating_17fluctuating, data = All_linemeans)
summary(Corr_OffspringPerMating_17Constant_17Fluctuating)


#Body size

#24 constant vs 24 fluctuating
Corr_wings_24Constant_24Fluctuating <- lm(Mean_wings_24constant ~ Mean_wings_24fluctuating, data =
All_linemeans)
summary(Corr_wings_24Constant_24Fluctuating)

#24 constant vs 17 constant
```

```
Corr_wings_24Constant_17Constant <- lm(Mean_wings_24constant ~ Mean_wings_17constant, data =
All_linemeans)
summary(Corr_wings_24Constant_17Constant)

#24 constant vs 17 fluctuating
Corr_wings_24Constant_17Fluctuating <- lm(Mean_wings_24constant ~ Mean_wings_17fluctuating, data =
All_linemeans)
summary(Corr_wings_24Constant_17Fluctuating)

#24 fluctuating vs 17 constant
Corr_wings_24Fluctuating_17Constant <- lm(Mean_wings_24fluctuating ~ Mean_wings_17constant, data =
All_linemeans)
summary(Corr_wings_24Fluctuating_17Constant)

#24 fluctuating vs 17 fluctuating
Corr_wings_24Fluctuating_17Fluctuating <- lm(Mean_wings_24fluctuating ~ Mean_wings_17fluctuating, data
= All_linemeans)
summary(Corr_wings_24Fluctuating_17Fluctuating)

#17 constant vs 17 fluctuating
Corr_wings_17Constant_17Fluctuating <- lm(Mean_wings_17constant ~ Mean_wings_17fluctuating, data =
All_linemeans)
summary(Corr_wings_17Constant_17Fluctuating)
```